UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
REGION IX
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August 15, 1995

Dave Song
Department of the Navy
Engineering Facilities Activity, West
900 Commodore Way, Building 101
San Bruno, California 94066-0720

Subject: Draft Final Phase 1B Ecological Risk Assessment Quality Assurance Project Plan, Hunters Point Naval Shipyard

Dear Mr. Song:

Enclosed please find the Environmental Protection Agency's (EPA's) comments regarding the Draft Final Quality Assurance Project Plan dated July 5, 1995.

If you have any questions regarding these comments, please call me at (415) 744-2410.

Sincerely,

Sheryl Lauth
Remedial Project Manager

Attachment

cc w/o attachment: Cyrus Shabahari, DTSC
Rich Hiatt, RWQCB
Richard Powell, Navy

ENVIRONMENTAL PROTECTION AGENCY COMMENTS
REGARDING THE QUALITY ASSURANCE PROJECT PLAN
FOR HUNTERS POINT NAVAL SHIPYARD PHASE 1B EFFORT

General Comments:

There is a concern that the analytical methods proposed in the QAPjP may not provide the information needed to evaluate possible ecological risk. The CLP methods for chemical analysis enumerated in the QAPjP may not provide low enough limits to detect certain contaminants of concern in San Francisco Bay sediments. Several procedures proposed for the Development Abnormality Toxicity Test with Strongylocentrotus purpuratus should be modified. It is suggested that the Standard Operating Procedure be replaced by the new EPA draft protocol, which accompanies this review.

In addition, it is not clear that the logistics of sample work-up, especially the preparation and use of pore water, have been fully analyzed. The Work Plan, which calls for a large number of sediment samples to be collected, also requires analysis of the pore water from those sediments. It is most important that the laboratory or laboratories contracted to perform these analyses have the capacity to produce and process the large volumes of pore water in a timely manner. Alternatively, methods using smaller volumes of sample, provided they achieve the required detection limits, should be researched.

Specific Comments:

1. Section 1.0: Introduction. This section contains the general statements regarding intention to use EPA CLP methods for chemical analysis of sediment, pore water and tissue. Unmodified CLP methods may not be appropriate for achieving meaningful detection limits for marine sediments. This issue should be discussed briefly in the introduction and more fully in the appropriate sections. The echinoderm bioassay protocol should reference the latest EPA version.

2. Section 3.5: Representativeness. In addition to the definition of representativeness presented here, a brief discussion of the method by which it was determined that this objective is being met by the sampling design should be included here.

3. Section 3.6: Comparability. The statement that levels of precision, accuracy and completeness are listed in Appendix A is inaccurate; only precision and accuracy objectives are included. This statement or the Appendix should be edited for consistency.

4. Section 4.0: Sampling Procedures. Several items related to Tables 1, 2, 4 and 5 need to be clarified or edited.

A. Table 1: Analytical Methods. Table 1 includes only the chemical analyses. The bioassays should either be included in this table or in a separate table (1B).

B. Table 2: Sample Container, Holding time and Preservative Requirements for Sediment Samples, Ammonia. It is not clear whether the sediment should be chilled and preserved for up to 28 days, chilled or preserved and kept at another temperature, or chilled and preserved prior to analysis. The treatment of the sediment for the analysis of ammonia may need to be clarified in a Standard Operating Procedure.

C. Table 4: Sample Container, Holding Time and Preservative Requirements for Tissue Residue Analysis. In the Note b, which refers to Sample Containers "G", is defined as "Glass jars with room left for freezing water." The note is ambiguous as stated and should be rewritten to include the concept of leaving headspace in the jars to allow for expansion of water in the sample.

D. Table 5: Sample Container, Holding time and Preservative Requirements for Sediment and Pore Water Bioassays. It is suggested that the echinoderm development test reference the most recent EPA protocol.

5. Section 8.0: Analytical Procedures and Reporting Limits. There are notes on several tables in this section that need clarification.

A. Table 11-14: Contract-Required Quantitation Limits (CRDL)] All these tables contain notes referring to the California maximum concentration limits or maximum contaminant level (both sets of terms are used, presumably referring to the same numbers), but these are not referenced, nor are they discussed in the text. In the Bay Protection and Toxic Cleanup Program QAPjP, prepared by the State Water Resources Control Board (July 1994), the detection limits for other analytes than those noted are lower than those listed in these tables. A discussion of the apparent discrepancy should be included in this section.

B. Table 14. The sediment and tissue CRDL column units are given as mg/kg, but the legend at the bottom of the table reads μm/kg. This inconsistency should be corrected.

C. Table 15: Miscellaneous Analyses CRDL. The units mg/kg should be written out in the legend.

6. Section 8.10.3: Echinoderm Development Test. The references for this test should be up-dated. The Chapman and Denton 1995 EPA protocol should be followed, as it is eliminates the several issues that are problematic in the current SOP, as discussed in item 10.

7. Section 9.1: Field Quality Control Samples. The need for temperature blanks should be discussed in this section.

8. Section 9.1.1: Field Duplicate Samples. For consistency, the need to collect duplicate samples of pore water and tissue should be discussed in this section, as Table 16: Field Quality Control Samples includes these media as well as sediment.

9. Appendix D: Standard Operating Procedure (SOP) for Determination of Sediment Biological Oxygen Demand (BOD), Section 3.4: Analytical Procedures. It is not clear in the directions under the second bullet which of the two methods for determining BOD is recommended. In the third bulleted paragraph, the instructions refer to analysis of a second sample. In the present context, it should read blank sample.

10. Appendix E: SOP for 48- to 96-H Development Abnormality Toxicity Test with Strongylocentrotus purpuratus. Because there are several issues in the protocol as written that need clarification or modification, it is suggested that the 1995 EPA protocol be used in its place.

A. Section 6.0: Bioassay Procedure. The density of the inoculum, 2000 organisms/20mL test chamber seems very high. Densities on the order of 30 organisms/mL are more common for development tests in small volumes.

B. Section 7.0: Daily Monitoring of the Tests. Taking water quality in a small test volume is difficult and may introduce contamination. A water quality blank should be made up for this purpose. Measuring ammonia at the end of the test is problematic because of the small total volume of test samples. Taking the ammonia concentration of the sample at the beginning of the test may be sufficient.

C. Section 9.0: Test Completion. The directions in the second paragraph seem to require compositing the replicates before counting the larvae. This would mean that true replication would be lost. The replicates should not be mixed; each replicate should be counted separately.

11. Appendix H: SOP for Conducting Sediment Pore Water Toxicity Test with the Luminescent Bacteria Photobacterium phosphoreum, Section 5.3: Osmotic Adjustment. The sentence beginning "NaCl reacts minimally..." does not make sense as it is written and needs to be edited.

12. Appendix I: SOP for Preparation of Tissue for Analysis.

A. Section 2.3: Preparation of Tissue Samples. This section needs to be edited and should be expanded to include more specific instructions as to how to avoid sample contamination.

B. Section 2.5: Tissue Preparation. There should be some

discussion concerning how to avoid possible contamination of the sample as it is processed in a grinder.

ATTACHMENT A

SEA URCHIN (*Strongylocentrotus purpuratus*) EMBRYO-LARVAL DEVELOPMENT TEST METHOD

15.1 **SCOPE AND APPLICATION**

15.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the developing embryos of the purple sea urchin, *Strongylocentrotus purpuratus* during a 72-h static exposure. The effects include the synergistic, antangonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

15.2 **SUMMARY OF METHOD**

15.2.1 The method provides the step-by-step instructions for performing a 72-h static test using the early development of the purple sea urchin, *Strongylocentrotus purpuratus* to determine the toxicity of substances in marine and estuarine waters. The test endpoint is normal larval development.

15.3 **INTERFERENCES**

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 **SAFETY**

15.4.1 See Section 3, Health and Safety

16.5 **APPARATUS AND EQUIPMENT**

16.5.1 Tanks or aquaria for holding adult sea urchins, e.g. standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 12°C), with appropriate filtration and aeration system.

16.5.2 Air pump, air lines, and air stones -- for aerating water containing adults (for static systems and emergency aeration for flow-through systems).

16.5.3 Refractometer -- for determining salinity.

16.5.4 Hydrometer(s) -- for calibrating refractometer.

16.5.5 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

16.5.6 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

16.5.7 pH and DO meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of these two parameters, portable, field-grade instruments are acceptable.

16.5.8 Standard or micro-Winkler apparatus -- for determining DO (optional).

16.5.9 Balance, Analytical, capable of accurately weighing to 0.0001 g -- for weighing reference toxicant.

16.5.10 Reference weights, Class S -- for checking performance of balance.

16.5.11 Cubitainers, beakers, or similar containers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes. Strong solutions of NaOH and formaldehyde should not be held for several month periods in Cubitainers: interaction or leaching into solutions of 0.1 N or 1 N NaOH used for pH adjustment of dilution water has caused poor egg fertilization; formaldehyde similarly stored has induced abberant partial membrane elevation in eggs.

16.5.12 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

16.5.13 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

16.5.14 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.15 Wash bottles -- for dilution water; for topping off graduated cylinders.

16.5.16 Wash bottles -- for reagent water; for rinsing small glassware and instrument electrodes and probes.

16.5.17 Constant temperature chamber or water bath for keeping dilution water supply and egg and embryo stock suspensions at test temperature prior to the test.

16.5.18 Water purification system -- Millipore Super-Q, deionized water (DI) or equivalent.

16.5.19 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

16.5.20 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

16.5.21 Compound microscope -- for examining gametes, counting sperm cells (200-400x), eggs and embryos and (100x), and examining larvae. Dissecting scopes are sometimes used to count eggs at a lower magnification. One piece of equipment worthy of a special mention is an inverted microscope. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

16.5.22 Hemacytometers, Neubauer -- for counting sperm.

16.5.23 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

16.5.24 Counter, two unit, 0-999 -- for recording sperm, egg, embryo, and larval counts.

16.5.25 Sedgwick-Rafter counting chamber -- for counting egg and embryo stock and examining larval development at the end of the test.

16.5.26 Centrifuge tubes, test tubes, or vials -- for holding semen.

16.5.27 Water bath, incubator, or room with temperature control -- for maintaining test solutions at 15°C during the test.

16.5.28 Graduated cylinders -- Class A, borosilicate glass or

non-toxic plastic labware, 50-1000 mL for making test solutions and sperm and egg stock solutions. (Note: not to be used interchangeably for gametes and test solutions).

16.5.29 Pipet bulbs and fillers -- PROPIPET^{R}, or equivalent.

16.5.30 Fume hood -- to protect the analyst from effluent, formaldehyde or glutaraldehyde fumes.

16.5.31 Glass stirring rods -- for mixing sand dollar semen suspensions.

16.5.32 Perforated plunger -- for maintaining homogeneous distribution of eggs and embryos during sampling and distribution to test chambers.

15.5.33 Enamel or plastic tray -- for optional spawning platform.

15.5.34 Nitex® screening (0.5mm mesh) --

16.6 **REAGENTS AND SUPPLIES**

16.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

16.6.2 Tape, colored -- for labelling containers.

16.6.3 Markers, water-proof -- for marking containers, etc.

16.6.4 Parafilm -- to cover graduated cylinders and vessels containing gametes.

16.6.5 Gloves, disposable -- for personal protection from contamination.

16.6.6 Data sheets (one set per test) -- for data recording (see Figures 1-4).

16.6.7 Acetic acid, 10%, reagent grade, in filtered (10μ) sea water -- for preparing killed sperm dilutions for sperm counts.

16.6.8 Formaldehyde, 10%, in seawater -- for preseving larvae.

16.6.9 Glutaraldehyde, 1% in seawater -- for preserving larvae.

Figure 1. Sample experiment set-up sheet.

SEA URCHIN EMBRYO DEVELOPMENT TEST

EXPERIMENT DESCRIPTION

Bioassay No.___________ Start Date________ Start time_______

Investigator___________ End Date__________ End Time_________

Sample Numbers	Sample	Concentration	Other Information

Figure 2. Sample worksheet for urchin spawning information.

SEA URCHIN DEVELOPMENT TEST

SPAWNING WORKSHEET

Bioassay no._____ Date _____

Spawning

No.	Injection time	Sex	Accepted? (Comments)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

Sperm density

#sperm counted=_____

_____

_____ mean=_____

_____

(mean)_____ x (5 x 10^6)=_____ sperm/mL

Egg dilution

eggs counted=_____

_____ mean=_____

_____

(mean)_____ x 100=_____ eggs/mL in stock

eggs/mL in stock ÷ 1,000= _____ Egg dilution factor

Figure 3. Sample worksheet for sea urchin fertilization information.

SEA URCHIN DEVELOPMENT TEST

FERTILIZATION WORKSHEET

Bioassay No. _____
Date_____

_____mL eggs used _____mL dilution water used

Fertilization and initiation

_____mL in egg dilution x 1,000 eggs/mL
= _____eggs in dilution

_____eggs in dilution x 500 sperm/egg
= _____sperm needed

_____sperm needed ÷ _____sperm/mL in sperm dilution=_____mL sperm dilution needed

Percent fertilized after 10 min _____

Time of inoculation_____

Figure 4. Sample data sheet for embryo microscopic examination.

SEA URCHIN DEVELOPMENT TEST: RESULTS

Bioassay No.______________ Date_______________

Counter_____________

16.6.10 pH buffers 4, 7, and 10 (or as per instructions of

instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

16.6.11 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents for modified Winkler analysis.

16.6.12 Laboratory quality assurance samples and standards for the above methods.

16.6.13 Reference toxicant solutions (see Section 4, Quality Assurance).

16.6.14 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

16.6.15 Haemo-Sol or equivalent cleaner for cleaning hemacytometer and cover slips.

16.6.16 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

16.6.17 Needles, 25 gauge -- for injecting KCl.

16.6.18 0.5 M KCl solution -- for inducing spawning.

16.6.19 Pipets, serological -- 1-10 mL, graduated.

16.6.20 Pipet tips for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

16.6.21 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

16.6.22 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

16.6.23 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

16.6.24 Coverslips -- for microscope slides.

16.6.25 Lens paper -- for cleaning microscope optics and hemacytometers.

16.6.26 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

16.6.27 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

16.6.28 20-mL glass scintillation vials with polypropylene caps-- It is recommended that the test be performed in 20 mL scintillation vials. After preservation, the samples may be counted inside the scintillation vial using an inverted microscope. New batches of vials should be tested for toxicity to controls before use in an actual assay. The test may be performed in other sized containers (beakers, tissue culture flasks, etc.), as long as the density of embryos is the same. A reference toxicant test must be performed on any alternate containers and this test must conform to the acceptability criteria set forth in Section 15.12.

16.6.28 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests.

16.6.28.1 Saline test and dilution water -- The salinity of the test water must be in the range of 32% to 36%. The salinity should vary by no more than ± 2% among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.6.29 HYPERSALINE BRINES

15.6.29.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually

require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

15.6.29.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100%) is used as a diluent, the maximum concentration of effluent (0%) that can be tested is 66% effluent at 34% salinity (see Table 1).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34% WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity %	Brine 60 %	Brine 70 %	Brine 80 %	Brine 90 %	Brine 100 %
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

15.6.29.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.29.4 Freeze Preparation of Brine

15.6.29.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

15.6.29.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100%. It is advisable not to exceed about 70% brine salinity unless it is necessary to test effluent concentrations greater than 50%.

15.6.29.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.29.5 Heat Preparation of Brine

15.6.29.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One

successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

15.6.29.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

15.6.29.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100% and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.29.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.29.6 Artificial Sea Salts

15.6.29.6.1 No data from sea urchin larval tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

15.6.29.7 Dilution Water Preparation from Brine

15.6.29.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse

effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

15.6.29.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100% and the test is to be conducted at 34%, 100% divided by 34% = 2.94. The proportion of brine is 1 part in 2.94 (one part brine to 2.94 parts reagent water). To make 1 L of dilution water at 34% salinity from a HSB of 100%, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

15.6.29.8 Test Solution Salinity Adjustment

15.6.29.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34% by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68%; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

15.6.29.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

15.6.29.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in %), the salinity of the effluent (SE, in %), and volume of the effluent to be added (VE, in mL). Then use

the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

15.6.29.8.4 This calculation assumes that dilution water salinity is 34 ± 2%.

15.6.29.9 Preparing Test Solutions

15.6.29.9.1 Ten mL of test solution are needed for each test container. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test containers. The remaining test solution can be used for chemistry.

15.6.29.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2% and a brine salinity of 66%, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test containers. The remaining test solution can be used for chemistry.

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT, BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

Step 1. Combine brine with reagent water or natural seawater to achieve a brine of 68% and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34%.

Step 2. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x%	Brine (68-x)%	Dilution Water* 34%
40	800 mL	800 mL	400 mL

Step 3. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

SERIAL DILUTION

Effluent Conc. (%)	Effluent Source	Dilution Water* (34%)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x%	Brine (68-x)%	Dilution Water* 34%
20	200 mL	200 mL	600 mL

10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

15.6.29.10 Brine Controls

15.6.29.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 15.6.29.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0%, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

15.6.30 TEST ORGANISMS, PURPLE URCHINS

15.6.30.1 Sea Urchins, Strongylocentrotus purpuratus (approximately 6 of each sex per test).

15.6.30.2 Adult sea urchins (Strongylocentrotus purpuratus) can be obtained from commercial suppliers or collected from uncontaminated intertidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.30.3 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.30.4 Although ambient temperature seawater is usually

acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14°C with a water temperature control device.

15.6.30.5 Food for sea urchins -- kelp, recommended, but not necessarily limited to, *Laminaria sp.*, *Hedophyllum sp.*, *Nereocystis sp.*, *Macrocystis sp.*, *Egregia sp.*, *Alaria sp.* or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60°C overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

15.6.30.6 Natural seawater (>30%) is used to maintain the adult animals and (≥32%) as a control water in the tests.

15.6.30.7 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.30.8 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (32%) and a gravel bed filtration system, are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.7 **EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE**

15.7.1 See Section 8, Effluent and Receiving Water Sampling

and Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 **CALIBRATION AND STANDARDIZATION**

15.8.1 See Section 4, Quality Assurance.

15.9 **QUALITY CONTROL**

15.9.1 See Section 4, Quality Assurance.

15.10 **TEST PROCEDURES**

15.10.1 TEST DESIGN

15.10.1.1 The test consists of at least five 20 mL replicates of at least five effluent concentrations (or one or more receiving water concentrations) plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

15.10.1.2 Effluent concentrations are expressed as percent effluent.

15.10.2 TEST SOLUTIONS

15.10.2.1 Receiving Waters

15.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 μm NITEX® filter and compared without dilution against a control. Using five replicates chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 450 mL or more of sample per test.

15.10.2.2 Effluents

15.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of

concentrations of 6.25%, 12.5%, 25%, 50%, and 100%. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. Therefore, USEPA recommends the use of the ≥0.5 dilution factor. If 100% HSB is used as the diluent, the maximum concentration of freshwater effluent that can be tested will be 66% at 34% salinity.

15.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

15.10.2.2.3 The volume of effluent required to

15.10.2.2.4 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.2.2.5 Just prior to test initiation (approximately 1 h), a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1°C) and maintained at that temperature during the addition of dilution water.

15.10.2.2.6 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labelled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

15.10.2.2.6 The series of test solutions, including controls and reference toxicants, should be prepared in sufficient quantity to allow for required and optional chemical analyses. A convenient volume is 100 mL in a 150 mL beaker.

15.10.2.2.7 At least 4 replicate vials are prepared for each test concentration, using 10 mL of solution in borosilicate glass

vials.

15.10.2.3 Dilution Water

15.10.2.3.1 Dilution water may be uncontaminated 1μm-filtered natural seawater or hypersaline brine prepared from natural seawater (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

15.10.2.4 Reference Toxicant Test

15.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

15.10.2.4.2 The preferred reference toxicant for sea urchins is copper chloride ($CuCl_2 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4, Quality Assurance). Another toxicant may be specified by the appropriate regulatory authority.

15.10.2.4.3 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test containers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

15.10.3 COLLECTION OF GAMETES FOR THE TEST

15.10.3.1 Spawning Induction

15.10.3.1.1 Pour seawater into each of six 100 mL beakers and place in 15°C bath or room. Allow to come to temperature. Select a sufficient number of sea urchins (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six sea urchins, three of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more sea urchins may be required.

15.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment. Place six to eight sea urchins on a clean tray covered with several layers of seawater moistened paper towels.

15.10.3.1.3 Inject each sea urchin with 0.5 mL of 0.5 M KCl, through the soft tissue surrounding the Aristotle's lantern. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Gently shake the animals once or twice to stimulate. Place animals back onto tray, oral side down. Females will release orange-colored eggs and males will release cream-colored semen. Note the time that each animal starts spawning (Figure 2). Place spawning females oral side up on the 100 mL beakers. Leave spawning males on tray for semen collection. Only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes.

15.10.3.1.2 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

15.10.3.2 Collection of Sperm

15.10.3.2.1 Semen should be collected dry, using either a Pasteur pipette or a 100 μL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 5 mL conical test tubes, stored in an ice water bath.

15.10.3.3 Viability of Sperm

15.10.3.3.1 A very small amount of semen from each male should be added to a drop of seawater in a counting chamber or a well slide. Examine the sperm for motility and use only sperm from animals where the motility is high.

15.10.3.4 Pooling of Sperm

15.10.3.4.1 Pool equal quantities of semen from each of the males that has been deemed good. At least 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL should be available.

15.10.3.5 Storage of Sperm

15.10.3.5.1 If sperm is not going to be used immediately, the tubes should be covered and stored in a refrigerator. Sperm should be used within four hours of collection. All collection of the eggs from the individual females should be completed before continuing with the sperm dilution.

15.10.4 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.4.1 Sperm Dilution

15.10.4.1.1 When ready to use sperm, mix by agitating the tube with a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15°C dilution water. Stir this solution thoroughly with a Pasteur pipette. A drop of egg solution from each female may be placed on a well slide and a small amount of sperm solution added to test fertilization. If no fertilization membrane forms on eggs from any female, then new gametes should be collected. Keep the sperm dilution covered and at 15°C until ready for use. This dilution should be used to fertilize the eggs within 1.5 hours of being made.

15.10.4.2 Sperm Density Determination

15.10.4.2.1 Take 0.5 mL subsample of the sperm solution and add it to 5 mL of 10% acetic acid in a 50 mL graduated cylinder, to kill the sperm. Bring the volume to 50 mL with dilution water. Mix by inversion and place one drop of the killed sperm solution onto each side of a hemocytometer. Let sperm settle for about 15 minutes. Count the number of sperm in 80 small squares on each side of the hemocytometer. If the counts for each side are within 80% of one another, then take the mean of those two counts. If the counts are not that close, then refill the hemocytometer, recount and take the mean of the four counts. Use

the following equations to determine sperm density and record the results on the spawning worksheet (Figure 2).

$$\#\text{sperm/mL} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion factor})(\text{mm3/mL})}{\text{number of squares counted}}$$

dilution=100
conversion factor=4000
mm^3/mL=1000
number of squares=80

Therefore:

$$\#\text{sperm/mL} = (\text{count})(5 \times 10^6) \qquad \text{(Equation 2A)}$$

15.10.5 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

15.10.5.1 Acceptability of Eggs

15.10.5.1.1 Place a small sample of eggs from each female in the counting chamber and examine eggs with the microscope. Look for the presence of significant quantities of immature or abnormal appearing eggs (germinal vesicle present, unusually large or small or irregularly shaped). Do not use the eggs from females having more than 10% abnormal eggs or from females whose eggs did not fertilize during the test in Section 15.10.5.1.

15.10.5.2 Pooling of Eggs

15.10.5.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. Pour the remaining solution through the Nitex® screen (to remove fecal material and other debris) into a 1 liter beaker. Repeat with each of the "good" females. Bring the volume up to about 600 mL with dilution water. Allow the eggs to settle to the bottom again. Siphon off about 400 mL of the overlying water and then bring back up to 600 mL with

dilution water.

15.10.5.3 Density of Eggs

15.10.5.3.1 Using a plunger, mix the egg solution well. While continuing to mix, remove a 10 mL sample and place in a 1 liter graduated cylinder. Bring the volume up to 1 liter with dilution water. Mix this dilution well and remove a 1 mL sample to a counting cell. Count all the eggs in the 1 mL sample. Repeat the process and take the mean of the two counts. Calculate the number of eggs per mL in the stock solution using Equation 3 and record the results.

# of eggs in count x 100= # eggs/mL in stock (Equation 3)

15.10.5.4 Dilution of Eggs

15.10.5.4.1 When using scintillation vials as the test chamber, the final concentration of eggs in the diluted stock must be 250 eggs/0.25 mL, which is equal to 1,000 eggs/mL. To calculate the dilution factor for the eggs, use Equation 4. (If larger test containers are used, the total number of eggs used will be greater and the stock solution density may be adjusted, but the final concentration of eggs in the test solutions must remain 25 eggs/mL).

# of eggs/mL in stock ÷ 1,000= Dilution factor (Equation 4)

15.10.5.4.2 The dilution factor must be greater than one. If not, concentrate the eggs and recount (starting at Section 15.4.5.3 The dilution factor minus 1 equals the number of parts of water that go with one part of eggs in the final dilution. For example: if the dilution factor were 5.3, then 4.3 parts of water would be used with 1 part eggs.

15.10.5.4.3 Make a dilution of the egg stock so that there is more than enough volume to perform the bioassay.

15.10.5.4.4 Fertilization of Eggs

15.10.5.4.4.1 The sperm to egg ratio needed for fertilization of the eggs is 500:1. Calculate the volume of sperm dilution

(Section 15.10.5.1) to add to the egg dilution, by using the following equations and record the results (Figure 3).

volume of egg dilution x 1,000 eggs/mL= total # of eggs in dilution (Equation 5A)

total # of eggs in dilution x 500 sperm/egg= # of sperm needed (Equation 5B)

# of sperm needed ÷ # sperm/mL in sperm dilution= mL of sperm solution (Equation 5C)

15.10.5.4.4.2 Add this volume of the sperm dilution to the egg dilution and mix gently with a plunger. Wait 10 min, then check for fertilization. If fertilization is not at least 90%, add an additional volume of the sperm dilution. Wait 10 min and recheck. If fertilization is still not 90%, then the test must be restarted with different gametes.

15.10.5.4.4.3 The test should be initiated within 1 hour of fertilization being achieved.

15.10.6 START OF THE TEST

15.10.6.1 Delivery of Fertilized Eggs

15.10.6.1.1 Gently mix the solution of fertilized eggs. Deliver 0.25 mL of egg solution to each vial, using an automatic pipette with the tip cut off to provide at least a 0.5 mm opening. Deliver the embryos into the test containers directly from the pipette, taking care not to touch the pipette to the test solution. The egg solution temperature must be within 1°C of the test solutions. Keep the eggs well mixed during the delivery procedure.

15.10.6.1.2 The embryos are incubated for 72 hours in the test containers at 15 ± 1°C at ambient light level.

15.10.7 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.7.1 The water temperature in the test chambers should be maintained 15 ± 1°C. The test salinity should be in the range of

34 ± 2°C. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.8 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.8.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

15.10.9 OBSERVATIONS DURING THE TEST

15.10.9.1 Routine Chemical and Physical Observations

15.10.9.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

15.10.9.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in the environmental chamber.

15.10.9.1.3 Record all the measurements on the data sheet.

15.10.9.2 Routine Biological Observations

15.10.9.2.1 Developing embryos do not need to be monitored during the test under normal circumstances.

15.10.10 TERMINATION OF THE TEST

15.10.10.1 Water Quality Measurements

15.10.10.1.1 Locate the vials designated for water quality analysis and place them in a separate location so they are not confused with samples intended for embryo examination.

15.10.10.1.2 All water quality measurements made at the start of the exposure should be repeated at the time of termination (See Section 15.10.9).

15.10.10.2 Sample Preservation

15.10.10.2.1 To terminate the test, add 1.0 mL of 37% (concentrated) buffered formalin to each sample to give a final formalin concentration of 4%. As an alternate fixative, 0.5 mL of 1.0% glutaraldehyde may be used, in each test container. Tightly cap and gently mix each container and store for later evaluation. (If the test is performed in larger containers, a 10 mL subsample of well mixed test solution is to be taken from each container and preserved).

15.10.10.3 Counting

15.10.10.3.1 It is recommended that the embryos be examined within one week of preservation. Longer storage times may also be used, but run the risk of sample degradation due to improper preservation. Samples can be counted directly from the scintillation vials using an inverted microscope. If an inverted scope is not available, then samples should be loaded into a Sedgewick-Rafter cell, as follows. The embryos should first be allowed to settle to the bottom of the sample container. All but about 1 mL of the overlying liquid should then be removed. All of the remaining liquid containing the embryos should then be transferred to the counting chamber. Whichever scope is used, the embryos should be examined at about 100x power. The first 100 embryos encountered are counted using a multi-unit handcounter to track normal versus abnormal larvae. Record the data by sample number on a data sheet (Figure 4).

15.10.10.4 Endpoint

15.10.10.4.1 Normal Larvae

15.10.10.4.1.1 Normally developed pluteus larvae have several distinctive characteristics:

1. The larvae should have a pyramid shape with a pair of skeletal rods that extend at least half the length of the long axis of the larvae (Figure 5D).

2. The gut should be differentiated into three parts (Figure 5E). If the gut appears lobed and constricts distally in specimens with an obstructed view (e.g., Figure 5D), then normal gut development may be inferred.

3. Development of post-oral arms has begun.

15.10.10.4.2 Abnormal Larvae

15.10.10.4.2.1 Larvae need only be scored as abnormal or normal to conduct the test, but the categories of abnormalities may be tracked as well. Abnormal larvae should fit into one of the following categories:

1. Pathological prehatched: Embryos at the single or multi-cell stage with the fertilization membrane still visible.

2. Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive degree of malformation or necrosis. Most of these larvae appear as dark balls of cells or dissociated blobs of cells.

3. Inhibited: larvae at the blastula or gastrula stage that have no gut differentiation or have no or underdeveloped skeleton. These larvae appear to be developing regularly, but are at a stage earlier than attained by control organisms (e.g., Figure 5A-C).

4. Gut abnormalities: larvae whose overall appearance is normal, but have guts that are lacking, undifferentiated, abnormally shaped or project outside of the larvae (exogastrulated).

5. Skeletal abnormalities: larvae whose overall appearance is normal, but have missing spicules, extraneous spicules or rods growing in abnormal directions. Note: Some larvae may exhibit a separation of the rods at the apex. This may be caused by preservation and should not be termed abnormal.

Since the test is started with already fertilized eggs, any unfertilized eggs that are encountered should not be counted as either normal or abnormal, but should be ignored.

15.11 SUMMARY OF TEST CONDITIONS

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE PURPLE URCHIN, *Strongylocentrotus purpuratus*, EMBRYO DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m_2/s$ (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	20 mL glass scintillation vials
8. Test solution volume:	10 mL
9. No. replicate chambers per concentration:	4
10. Dilution water:	Uncontaminated 1-μm-filtered natural seawater or; hypersaline brine plus reagent water
11. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor:	Effluents: ≥0.5 Receiving waters: 100% receiving water and a control
13. Test duration:	72 ± 2 hr
14. Endpoint:	Normal development
15. Test acceptability criteria:	≥80% normal shell development in the controls; must achieve a %MSD of <25%

16. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
17. Sample volume required:	500 mL per test

15.12 **ACCEPTABILITY OF TEST RESULTS**

15.12.1 Test results are acceptable only if all the following requirements are met:

(1) larval normality must be at least 80% in the controls.

(2) the minimum significant difference (%MSD) is ≤ 20% relative to the controls.

15.13 **DATA ANALYSIS**

15.13.1 GENERAL

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single Laboratory Precision

15.14.1.1.1 Data on the single-laboratory precision of the development test using copper as a reference toxicant is provided in Table 4. The NOEC varied by only one concentration interval indicating good precision. The coefficient of variation for the EC50 and EC25 were 22% and 21% indicating acceptable precision.

15.14.1.2 Multi-Laboratory Precision

15.14.1.2.1 Data on the multi-laboratory precision of the development test using copper as a reference toxicant is provided in Table 5. The NOEC for laboratory's A and B were identical. The difference in NOEC observed for lab C was probably due the wide range of concentrations used (See Footnote 4). The coefficient of variation for the EC50 was 39%, indicating acceptable interlaboratory precision.

15.14.2 ACCURACY

15.14.2.1 The accuracy of toxicity tests cannot be determined.

Table 4. SINGLE-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *Strongylocentrotus purpuratus*, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT[1].

Test Number	NOEC (µg/L)	EC50 (µg/L)	EC25 (µg/L)
1	10.0	19.4	15.1
2	10.0	18.3	15.4
3	5.6	10.8	9.0
4	5.6	14.3	11.0
5	5.6	16.8	12.9
n:	5	5	5
Mean:	NA	15.9	12.7
CV(%):	NA	22	21

[1] Tests performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

TABLE 5. MULTI-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *Strongylocentrotus purpuratus*, DEVELOPMENT TEST WITH COPPER (CUμG/L) SULFATE AS A REFERENCE TOXICANT.[1]

Lab	NOEC (μg/L)	EC50 (μg/L)
A[2]	10	22.5
B[3]	10	15.2
C[4]	1.8	10.1
n:	3	3
Mean:	NA	15.9
CV(%):	NA	39

[1] Data from labs A and B are from an interlaboratory study using split reference toxicant samples and dilution water. Test performed in August, 1993. Test duration was 72 hr. Concentrations were 3.2, 5.6, 10, 18 and 32 μg/L.

[2] Test performed by Southern California Coastal Water Research Project, Westminster, CA.

[3] Test performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

[4] Test performed by MEC Analytical Systems, Inc., Tiburon, CA. Test performed in April, 1994. Test duration was 96 hr. Concentrations were 0.1, 0.32, 1.8, 18 and 56 μg/L.

APPENDIX A. SEA URCHIN DEVELOPMENT: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2%. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 g of $CuCl_2 \cdot 2H_20$ to 1 liter of reagent water.

D. Prepare copper reference toxicant series of 0 (control), 3.2, 5.6, 10.0, 18.0, 32.0 µg/L by adding 0, 0.32, 0.56, 1.0, 1.8 and 3.2 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water. Add 10 mL of test solution each vial.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen of each test concentration.

F. Randomize numbers for test containers and record the container numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test containers in a water bath or environmental chamber set to 15°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate container) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARTION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Place six 100 mL beakers of dilution water in 15°C water bath or room. Select 6-8 sea urchins and place on tray covered with seawater moistened paper towels. Induce spawning by injecting each sea urchin with 0.5 mL of 0.5 M KCl. Place animals back onto tray, oral side down.

C. When spawning begins, note time that each animal begins spawning. Leave males on tray for semen collection. Place spawning females oral side up on 100 mL beakers. Do not collect gametes more than 15 min after spawning begins.

D. Collect semen using either a Pasteur pipette or a 100 µL autopipette. Pipette semen from each male into a separate 5 mL conical test tube, stored in an ice water bath.

E. Check for the motility of sperm from each male.

F. Pool semen by pipetting equal amounts from each "good" male to another centrifuge tube. At least 0.025 mL should be taken from each male and a total of at least 0.05 mL should be available. Cover the tube and store in a refrigerator until ready for use.

G. Finish collecting eggs before diluting semen.

H. Mix pooled semen by agitating on a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15°C dilution water. Stir thoroughly with a Pasteur pipette. Test eggs from each female to determine if they can be fertilized.

I. Take 0.5 mL subsample of sperm dilution and add to 5 mL of 10% acetic acid in a 50 mL graduated cylinder. Bring to 50 mL with dilution water. Mix well by inversion and load a drop into each side of hemocytometer. Count the sperm in 80 small squares. Calculate the sperm density using Equation 2A.

J. Examine sample of eggs from each female. Do not use the eggs from any female whose eggs appear abnormal or that did not fertilize in Section G.

K. Decant water from eggs of each usable female and pour through Nitex® screen into a 1 liter beaker. Bring volume up to about 600 mL with dilution water. Allow to resettle, siphon about 400 mL of overlying water and bring back to 600 mL with dilution water.

L. Mix egg solution well and make an accurate 100x dilution using at least 10 mL of the egg solution. Mix the dilution well and count two different 1 mL subsamples in a counting cell. Use the mean of the two counts in Equation 3 to determine the density of the egg stock.

M. Use Equation 4 to determine the egg dilution factor and make dilution of eggs with dilution water.

N. Use Equations 5 A-C to determine the volume of the sperm dilution that is necessary to fertilize the egg dilution. Add the appropriate volume of sperm and after 10 minutes, check fertilization success.

O. Gently mix the fertilized egg solution with a plunger and deliver 0.25 mL of egg solution to each vial. Make sure that the pipette tip is cut off to provide at least a 0.5 mm opening. Keep egg solution well mixed during addition period.

P. Incubate the embryos for 72 hours at 15 ± 1°C.

Q. Test termination and analysis

R. Perform water quality measurements as at the start.

S. After 72 hours, add 1.0 mL of 37% buffered formalin or 0.5 mL of 1.0% glutaraldehyde to each test container. Tightly cap and gently mix each vial.

T. Examine each sample with a microscope and determine the percentage of normally developed embryos.

U. Analyze the data.

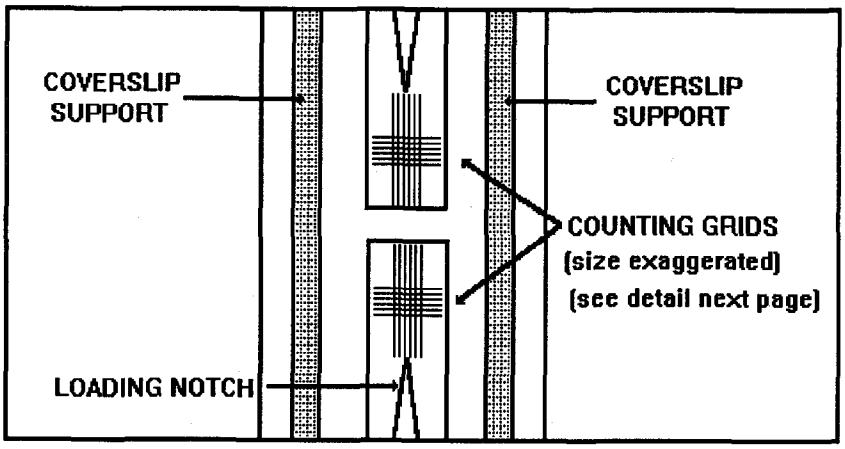
V. Include standard reference toxicant point estimate values in the standard quality control charts.

Figure 5. Stages of sea urchin embryo development (modified from Kume and Dan 1957). A. blastula; B. gastrula; C. prism; D. pluteus (frontal view); E. pluteus (lateral view). *al.arm:* anterior lateral arm, *e:* esophagus, *i:* intestine, *st:* stomach, *std:* stomodaeum.

APPENDIX B. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

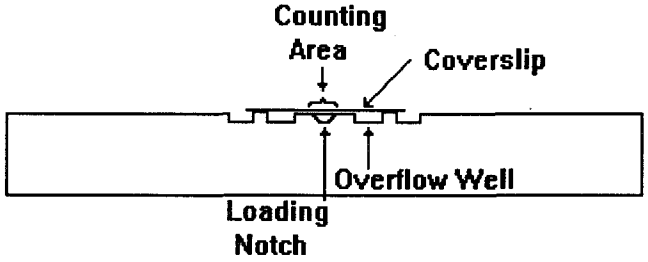
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

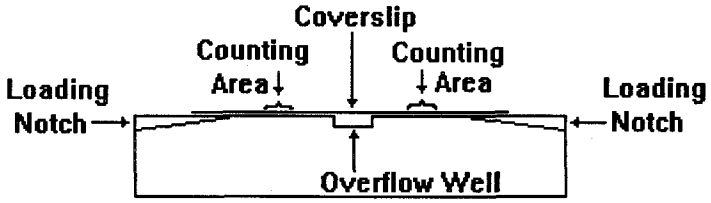


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

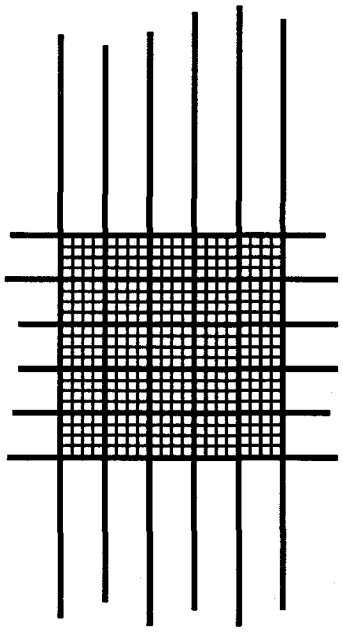
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g. blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



NEUBAUER HEMACYTOMETER GRID OF 400 SQUARES

If the full 400-squares of each grid is counted, this represents the number of sperm in 0.1 mm . Multiplying this value times 10 yields the sperm per mm (and is the source of the hemacytometer factor of 4,000 squares/mm). If this product is multiplied by 1,000 mm /cm , the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{(\text{dilution})\ (4{,}000\ \text{squares/mm}^3)\ (1{,}000\ \text{mm}^3/\text{cm}^3)\ (\text{cell count})}{(\text{number of squares counted})}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

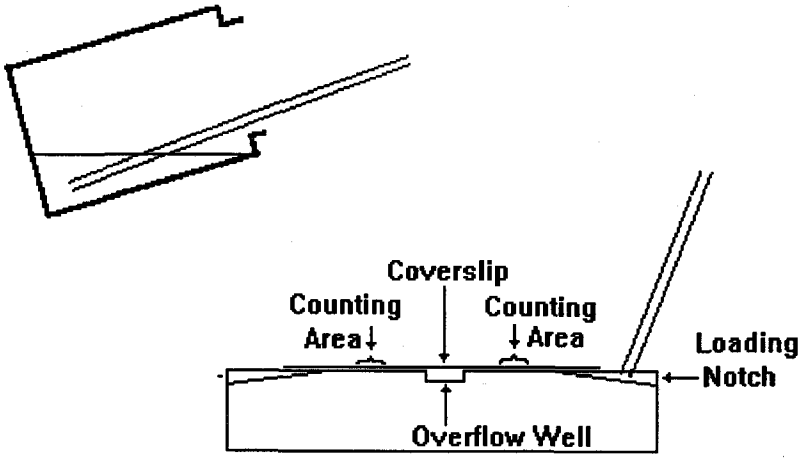
$$= 20{,}000{,}000{,}000\ \text{cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

Obviously, if the sample is not homogeneous, subsamples can vary

in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

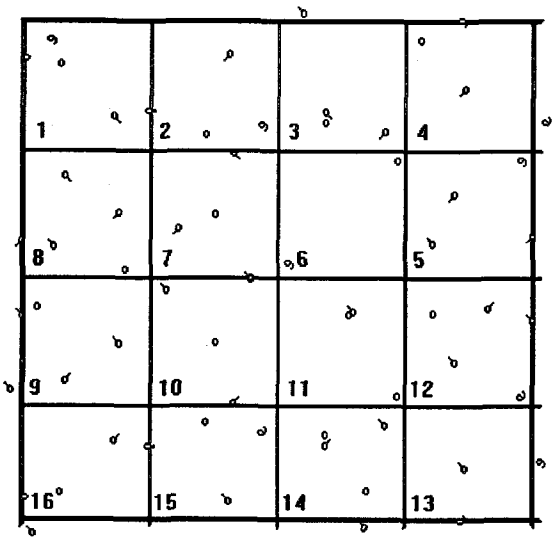
Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.



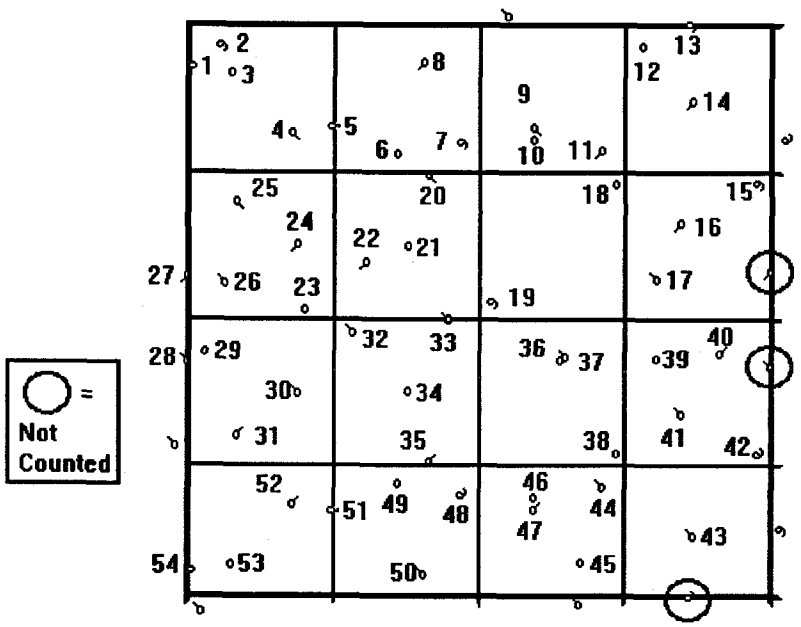
The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below).

Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear <u>on</u> lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

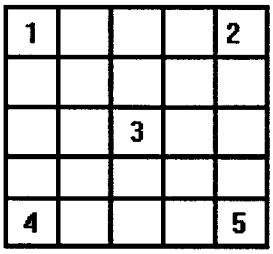
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

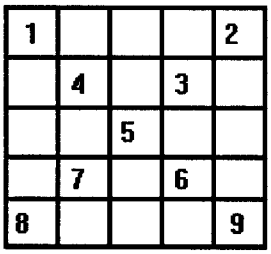
1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

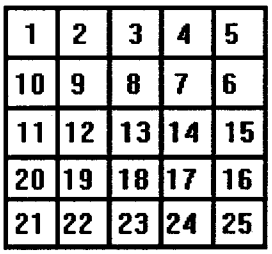
3. If the number is 20 or greater, count 5 large squares using pattern no. 1.



Pattern no. 1



Pattern no. 2



Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.

References

Kume, M. and K. Dan. 1957. Invertebrate Embryology. U.S. Department of Commerce, National Technical Information Service, No. TT67-58050, Washington, D.C. 605 pp.